Therapeutic Studies in NZB/W Mice

II. Relative Efficacy of Azathioprine, Cyclophosphamide and Methylprednisolone

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Five-month-old female NZB/W mice were treated with daily injections of azathioprine, cyclophosphamide or methylprednisolone. Treatment was continued for 2 or 3 months at different dosages. Renal histology, proteinuria, antibodies to DNA and survival were studied. All drugs were found to modify the disease favorably. At low doses (1.5 mg/kg/day) azathioprine was most effective. Cyclophosphamide was superior at higher doses (4.5 mg/kg/day). Different two-drug combinations were also compared. The combination of cyclophosphamide plus methylprednisolone was most effective. Combined therapy increased survival without increasing toxicity.

There has been no controlled study in human renal disease comparing the efficacy of commonly used modes of therapy: corticosteroids, alkylating agents (generally cyclophosphamide) and purine analogs (generally azathioprine or its active metabolite 6-Mercaptopurine). New Zealand mice, particularly NZB/NZW F_1 (NZB/W) hybrid females, provide a convenient model in which to compare drugs in conjunction with similar studies in man. These mice develop a disease resembling human systemic lupus erythematosus (1) characterized by antibodies to nucleic acids and immune-complex glomerulonephritis from which they die after the age of 8 months (2, 3).

It has previously been found that cyclophosphamide (4) and methylprednisolone, with or without azathioprine (5), are effective in delaying this murine renal disease. The first report in this series compared the combined results of low dose single, double and triple agent therapy, and concluded that triple agent therapy was superior (6). The present report details the individual effects of cyclophosphamide, azathioprine and methylprednisolone at different doses upon the autoimmune nephritis of NZB/W mice. The three possible two-drug combinations were similarly studied.

MATERIALS AND METHODS

Animals. NZB/W female mice were obtained by NZB female × NZW male matings from stock colonies maintained at the National Institutes of Health, USA. Experimental and control animals were housed in the same area and allowed food and water ad libitum.

Immunosuppressive agents. Methylprednisolone sodium succinate* cyclophosphamide† and azathioprine‡ were

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freshly dissolved in 0.9% sterile saline so that the drug could be given in a volume of about 0.3 ml. The azathioprine first had to be dissolved in 0.1 N NaOH, then diluted in 0.9% saline and pH adjusted from 7.4 to 7.6 with 0.1 N HCl.

Experimental Design. Five-month-old female NZB/W mice were randomly allocated to different treatment groups, including a "no treatment group." All injections were intraperitoneal. Three experimental protocols were followed: a) Low dose: In the first experiment each drug was given for 3 months at 1.5 mg/kg/day, either singly or in combination; b) High dose: Three times as much of each drug, 4.5 mg/kg/day, was given separately in the second experiment, again for 3 months and c) In the last experiment mice received either cyclophosphamide, 3 mg/kg/day, methylprednisolone, 10 mg/kg/day or their combination. Therapy was continued for 2 months only. Other groups of mice received azathioprine 10 mg/kg with or without each of the other agents. These azathioprine-treated animals suffered from drug toxicity with wasting and death so that therapeutic efficacy could not be determined.

In Experiments 1 and 3 some of the mice were randomly sacrificed for renal histologic examination at eight months of age. The remaining mice were retained for survival studies. In addition, antibodies to DNA, proteinuria, body weight, hematocrit and white blood cell count were determined at 7 or 8 months of age.

Proteinuria Determination. Protein concentration in freshly expressed urine from individual animals was detected using "Albustix" (tetrabromphenol inpregnated paper). A positive urinary protein concentration was considered to be 100 mg% or more.

Anti-DNA Antibody Determination. Anti-DNA antibodies were measured by the ammonium sulphate precipi-

Table 1. Comparison of Different Regimens for Treatment of Female NZB/NZW Mice Daily for 3 Months Starting at 5 Months of Age

Treatment	No. of mice	Renal histologic score±SE*	Percent of Mice with proteinuria (≥100 mg %)		DNA bound (Mean %±SE)	Median survival (days)
		8 mo	7 mo	8 mo	8 mo	
None	27	2.64±.06	60	60	64±6	289
Experiment 1 (1.5 mg/kg/day)						
Single-drug regimen						
Azathioprine	22	1.83±.10†	29	43	52±6	350§
Cyclophosphamide	22	2.03±.18†	29	50	58±4	315
Methylprednisolone	21	2.21±.17†	15‡	31	51±4	302
Two-drug regimen						
Azathioprine + methylprednisolone	22	1.90±.09†	21	36	58±7	332‡
Cyclophosphamide + azathioprine	21	2.09±.10†	15‡	39	46±4†	329
Cyclophosphamide + methylprednisolone	22	1.68±.07†‖	7‡	21	49±4†	>365§¶
Experiment 2 (4.5 mg/kg/day)						
High dose						
Azathioprine	8	—	38	38	46±6†	291
Cyclophosphamide	8	—	13‡	13‡	38±5†	360§
Methylprednisolone	8	—	13‡	38	42±5†	309

*Eight mice in each group were sacrificed at 8 months for renal histology. The remainder were followed for survival.

†$P<0.05$ compared with controls (t test)

‡$P<0.05$ compared with controls (χ^2 test)

§$P<0.05$ compared with controls (Kolmogorov-Smirnov test)

‖$P<0.05$ compared with single agents (t test)

¶$P<0.05$ compared with single agents (Kolmogorov-Smirnov test)

tation assay using ^{14}C-labeled KB cell DNA is antigen, as previously described (7). Individual sera were diluted 1:10, 35% Ammonium Sulphate was used, and results expressed as the mean percentage bound.

Histologic Study. Upon sacrifice, kidneys were removed, promptly fixed in 10% neutral buffered formalin, imbedded in paraffin and sectioned at 3 to 4 μ. Hemotoxylin and eosin, periodic acid-Schiff (PAS) and Masson's trichrome stains were used.

Fifty glomeruli in each kidney specimen were graded on a 0 to 3 scale, as previously described (6). Grade 0 glomeruli contained no identifiable lesion. Grade 1 changes consisted of a mild increase in the amount of PAS positive mesangial matrix material and slight focal hypercellularity of the mesangial regions. Grade 2 changes were similar but more severe, usually with diffuse increase in mesangial matrix and increased cellularity of the mesangial region. Grade 3 changes were the most advanced and were characterized by irregular basement membrane thickening, hypercellularity of the mesangial region and increased mesangial sclerosis. The average histologic score for each specimen was the arithmetic mean of the 50 separate scores for individual glomeruli.

RESULTS

Low-Dose Therapy

A summary of the effects of three different drugs given separately at 1.5 mg/kg/day is presented in the upper part of Table 1. The average renal histologic score was lowest, indicating least disease, in the azathioprine-treated group. Both cyclophosphamide and methylprednisolone also significantly reduced histologic glomerulonephritis. Proteinuria was reduced by all three drugs. This was more marked at 7 months than at 8 months. The only statistically significant reduction was by methylprednisolone at 7 months. Anti-DNA antibodies were reduced in all three drug groups but these reductions were not statistically significant. At low dose, median survival was significantly prolonged only by azathioprine. Methylprednisolone, most effective in reducing proteinuria and anti-DNA antibodies, was least effective in altering the kidney histologically and prolonging survival. The order of effectiveness in prolonging survival was the same as that for histologic improvement: azathioprine first, cyclophosphamide next and methylprednisolone last. In fact, the correlation between histologic score and survival for all groups in Table 1 was highly significant ($P < 0.001$, Spearman rank correlation test).

Among the two-drug regimens, one combination—ie, methylprednisolone plus cyclophosphamide, was superior to the other two (middle part of Table 1). The average renal histologic score of animals treated with low dose cyclophosphamide plus methylprednisolone was significantly lower than average single agent scores; the other double agent regimens were not. Mice treated with cyclophosphamide plus methylprednisolone also had the least proteinuria and greatest survival among the two-drug combinations. Cyclophosphamide combined with either methylprednisolone or azathioprine significantly reduced anti-DNA antibodies.

High-Dose Therapy

At a dose of 4.5 mg/kg/day all three drugs significantly reduced anti-DNA antibodies (lower part of Table 1). Proteinuria was significantly reduced only in the cyclophosphamide group. Similarly, median survival was significantly prolonged only in cyclophosphamide-treated animals. The effectiveness of the combination of cyclophosphamide and methylprednisolone was reproduced at another dosage level (Table 2). All three regimens reduced histologic glomerulonephritis. Histologic scores from combined therapy were significantly lower than average single agent scores. Combined therapy also was more effective in reducing proteinuria, anti-DNA antibodies and mortality. The renal histology was not as good as after low-dose therapy, presumably because of the 1 month interval between stopping drug therapy and histologic evaluation. Survival was greater in the higher dose groups than in low dose groups (compare Tables 1 and 2).

Toxicity

Death from renal disease in NZB/NZW

Table 2. Comparison of High Dose Cyclophosphamide (3 mg/kg) and Methylprednisolone (10 mg/kg) and the Combination for 2 Months

Treatment	No. of mice	Renal histologic score±SE*	Percent of mice with proteinuria≥100 mg%	DNA bound (Mean%±SE)	Median survival (days)
		8 mo	7 mo	7 mo	
None	20	2.68±.07	60	46±6	297
Cyclophosphamide (3.0mg/kg/day)	20	2.22±.13†	10§	39±5	374‖
Methylprednisolone (10mg/kg/day)	19	2.26±.19†	11§	34±4	373‖
Cyclophosphamide + methylprednisolone	19	1.98●.10‡	5§	18±4‡	410‖

*Ten mice in each group were sacrificed at 8 months for renal histology. The remainder were followed for survival.

†$P<0.05$ compared with controls (t test)

‡$P<0.05$ compared with single agent (t test)

§$P<0.05$ compared with controls (χ^2 test)

‖$P<0.05$ compared with controls (Kolmogorov-Smirnov test)

¶$P<0.05$ compared with single agents (Kolmogorov-Smirnov test)

mice is characteristically preceded by 2 to 3 days of generalized anasarca. Drug toxicity leading to death may therefore be distinguished. In addition, weight loss and bone marrow suppression were monitored as indices of drug toxicity. Low doses of drug (1.5 mg/kg/day), given singly or in combination, led to no toxic deaths, significant weight loss or bone marrow suppression. At higher doses some toxicity was observed; 1 of 19 mice treated with methylprednisolone (10 mg/kg) and 1 of 20 mice treated with combined methylprednisolone (10 mg/kg) plus cyclophosphamide (3 mg/kg) died of apparent drug toxicity during the course of therapy. Otherwise, these drug regimens did not markedly reduce weight, hematocrit or white cell count.

Higher doses of azathioprine were associated with the most toxicity. One-fourth of the mice given 4.5 mg/kg/day of azathioprine and ½ of those treated with 10 mg/kg/day died of apparent drug toxicity during 3 months of therapy. By contrast, 4.5 mg/kg/day of cyclophosphamide was well tolerated, as was 4.5 or 10 mg/kg/day of methylprednisolone.

Mice were examined at least weekly for tumor formation. No malignancy was observed in any of the mice to 1 year of age.

DISCUSSION

This report details the comparative efficacy of cyclophosphamide, azathioprine and methylprednisolone in the therapy of glomerulonephritis in NZB/W mice. Treatment was started at 5 months of age, a time when these mice already had circulating antibodies to nucleic acids and gammaglobulin deposition in their kidneys. The study is therefore a therapeutic rather than preventive one, with therapy started early in the course of their clinical disease.

All three drugs were therapeutically useful when given in doses somewhat below toxic levels. Azathioprine was most effective at 1.5 mg/kg/day and least effective at 4.5 mg/kg/day. The best single agent programs were 3.0 to 4.5

mg/kg/day of cyclophosphamide and 10 mg/kg/day of methylprednisolone. Renal histology correlated very well with survival, whereas proteinuria and antibodies to DNA did not correlate as well.

Combined therapy proved more effective than single drug therapy for only one of the two-drug combinations. The combination of cyclophosphamide plus methylprednisolone resulted in significantly improved histology and survival compared with single drug therapy both at low and higher doses. The latter program resulted in a 40% increase in median survival. This enhanced survival was obtained despite only 2 months of treatment. It is possible that much longer survival would have been achieved had the drugs been continued indefinitely. On the other hand, continuous immunosuppression could lead to an increase in malignancy, especially after 1 year of age. We did not observe tumor formation before 1 year of age in the present series of experiments with short courses of treatment given and low doses used. Higher doses of azathioprine have been associated with lymphoid malignancy in NZB mice (8).

Although increased toxicity has been observed with the combination of very high doses of cyclophosphamide and methylprednisolone (9), at the low doses employed in the present study, no synergistic or even additive toxicity was observed.

Our results with combined cyclophosphamide plus methylprednisolone therapy are similar to the situation in human lupus where combined cyclophosphamide plus prednisone appeared effective (10). The present study is superficially at odds with the finding that azathioprine is ineffective in treating the Coombs positivity of NZB mice (8). Renal inflammation and Coombs positivity could easily respond very differently to a given drug. Azathioprine may not significantly reduce antibody production, but may sufficiently reduce renal inflammation to prolong life.

The efficacy of combined cyclophosphamide plus methylprednisolone therapy in treating NZB/NZW nephritis is in accord with the basic observation that combined drug therapy is particularly effective in producing immunosuppression (11). However, all of the agents have antiinflammatory as well as immunosuppressive actions (12–14). When given in low daily doses they may be exerting much of their effect through antiinflammatory mechanisms. In fact, mice so treated had relatively high levels of anti-DNA antibodies with significantly reduced histologic nephritis arguing against major immunosuppression. Studies of the degree of immune complex deposition during immunosuppressive therapy may help to answer this question.

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